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Dynamic EMT: a multi-tool for tumor progression

 Simone Brabletz^{*} , Harald Schuhwerk , Thomas Brabletz  & Marc P. Stemmler^{**} 

Abstract

The process of epithelial–mesenchymal transition (EMT) is fundamental for embryonic morphogenesis. Cells undergoing it lose epithelial characteristics and integrity, acquire mesenchymal features, and become motile. In cancer, this program is hijacked to confer essential changes in morphology and motility that fuel invasion. In addition, EMT is increasingly understood to orchestrate a large variety of complementary cancer features, such as tumor cell stemness, tumorigenicity, resistance to therapy and adaptation to changes in the microenvironment. In this review, we summarize recent findings related to these various classical and non-classical functions, and introduce EMT as a true tumorigenic multi-tool, involved in many aspects of cancer. We suggest that therapeutic targeting of the EMT process will—if acknowledging these complexities—be a possibility to concurrently interfere with tumor progression on many levels.

Keywords EMT; MET; ZEB1; ZEB2; SNAIL; SLUG; TWIST; cancer; invasion; metastasis; signaling pathways; cell plasticity; tumor stemness; hybrid EMT; partial EMT

Subject Categories Cancer; Cell Adhesion, Polarity & Cytoskeleton; Chromatin, Transcription & Genomics

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Introduction

Epithelial-to-mesenchymal transition (EMT) describes the transdifferentiation of stationary epithelial cells to a mesenchymal, motile phenotype and was initially observed in early development (Hay, 1995). Here, EMT contributes to embryonal processes like gastrulation, neural crest formation, or heart development (Thiery *et al*, 2009; Nieto *et al*, 2016). The program is also crucial for physiological processes like wound healing (Arnoux *et al*, 2008) and tissue homeostasis (Ahmed *et al*, 2006). Importantly, pathological reactivation of the EMT process plays a fundamental role in diseases like organ fibrosis or cancer progression to metastasis (Fig 1A), which is the focus of this review.

Cancer is an extremely complex and diverse disease not only varying between entities, but also within the same entity, between different subtypes, and even within subtypes. Notably, tumors

display not only spatial but also temporal heterogeneity within the same individual that can be elicited, e.g., via the occurrence of consecutive mutations and clonal evolution (McGranahan & Swanton, 2017). However, cancer cell plasticity, allowing continuous and reversible adaption to ever-changing conditions, is mediated by the EMT process that is not genetically fixed, depending on accumulating mutations, but is epigenetically orchestrated by signals from the microenvironment, rendering the whole program reversible (by activating mesenchymal–epithelial transition; MET) and highly dynamic (see overview in Fig 2).

The EMT program is mainly executed by a core set of EMT-activating transcription factors (EMT-TFs) including SNAIL (also SNAI1) and SLUG (also SNAI2), the basic helix–loop–helix factors TWIST1 (TWIST) and TWIST2 and the zinc finger E-Box binding homeobox factors ZEB1 and ZEB2. All these factors share the ability to repress epithelial genes like the E-cadherin encoding gene *CDH1* via binding to E-Box motifs in their cognate promoter regions (Nieto *et al*, 2016) as shown for SNAIL (Batlle *et al*, 2000; Cano *et al*, 2000), TWIST (Yang *et al*, 2004), ZEB1 (Eger *et al*, 2005), and ZEB2 (Comijn *et al*, 2001). In parallel, the EMT-TFs directly or indirectly activate genes associated with a mesenchymal phenotype, including *VIM* (Vimentin), *FN1* (Fibronectin), and *CDH2* (N-cadherin) (Nieto *et al*, 2016; Dongre & Weinberg, 2019) (Fig 1B). However, many functions are not shared, but executed by distinct EMT-TFs, as they differ in aspects like expression patterns or extremely different protein size and structure (Stemmler *et al*, 2019).

Beyond the “classical” EMT traits, the gain of motility, and invasive capacities, the widespread importance of EMT-TFs in cancer biology is indicated by additional pleiotropic functions (Brabletz *et al*, 2018). EMT-TFs have been shown to maintain stemness properties and to increase tumorigenicity, linking them to the concept of cancer stem cells (CSCs). Additionally, EMT-TFs are involved in DNA repair, the escape from senescence and apoptosis, therapy resistance, and immune evasion resulting in a pro-survival phenotype, providing an advantage under various types of stress conditions.

Altogether, the classical EMT functions together with the very diverse non-redundant context-dependent non-classical functions of EMT-TFs that are dynamically regulated also by the tumor microenvironment (TME) allow the cancer cells to permanently adapt to changing conditions (Puisieux *et al*, 2014). Consequently, therapeutic intervention with EMT/plasticity will provide the opportunity to fight many aspects of tumor progression at a single blow.

In this review, we summarize all these different EMT functions in cancer biology and highlight the resulting clinical implications.

Department of Experimental Medicine 1, Nikolaus-Fiebiger Center for Molecular Medicine, Friedrich-Alexander University of Erlangen-Nürnberg, Erlangen, Germany

*Corresponding author. Tel: +49 9131 85 29101; E-mail: simone.brabletz@fau.de

**Corresponding author. Tel: +49 9131 85 29101; E-mail: marc.stemmler@fau.de

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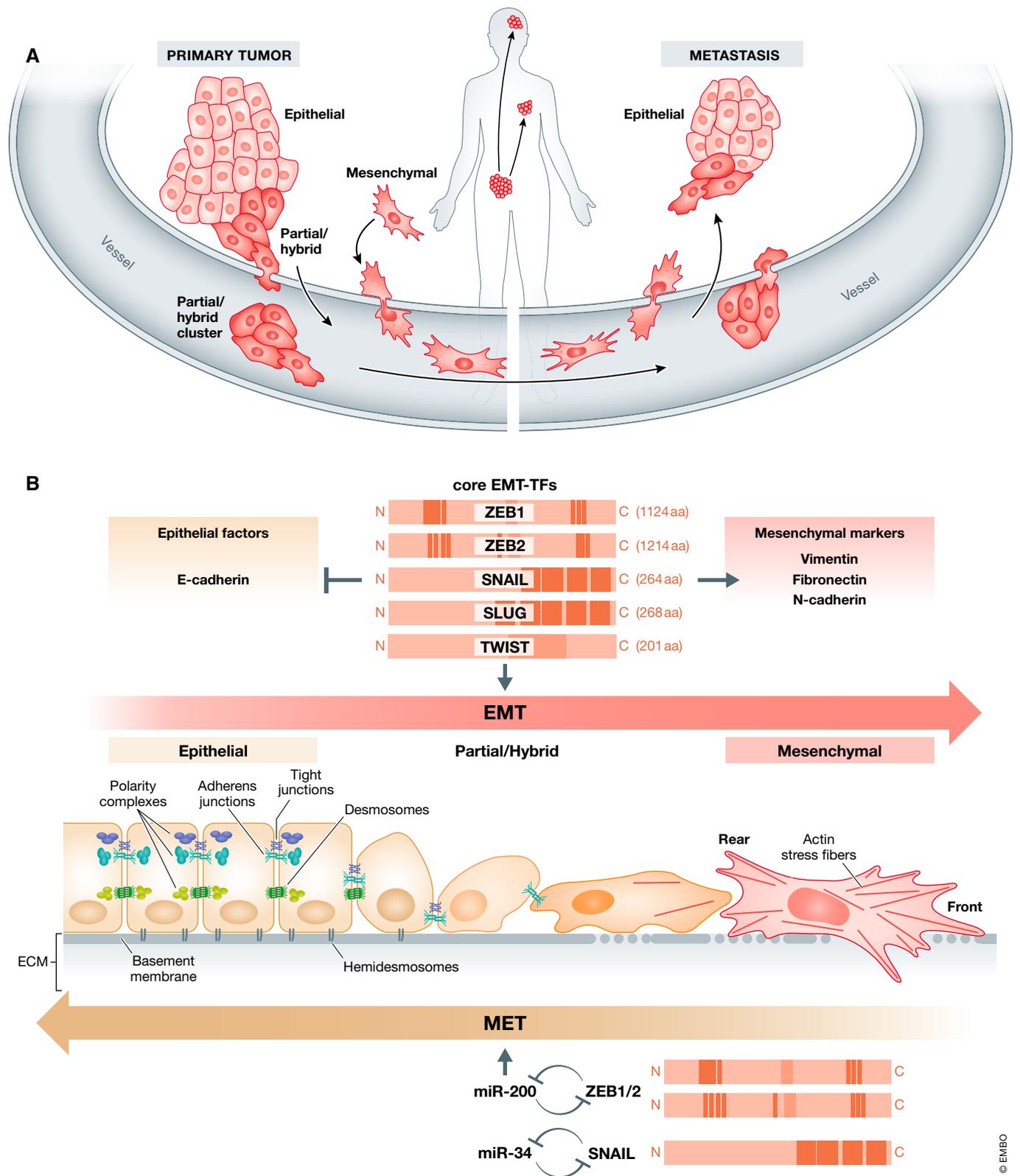


Figure 1.

Figure 1. Classical EMT functions and cancer.

(A) EMT frequently occurs at the invasive front of epithelial tumors, destroys the well-defined epithelial structures, and allows the cancer cells to migrate, invade the tissue, and intravasate in blood or lymphatic vessels. Tumor cells on their way through the body can travel as mesenchymal single cells, as cell clusters exhibiting partial EMT or as more epithelial cell clusters headed by a mesenchymal leader cell. At the secondary site, the cells extravasate and colonize the distant organ, where MET allows the outgrowth to macrometastases. (B) EMT is induced mainly by a set of transcription factors (EMT-TFs) like ZEB1, ZEB2, SNAIL, SLUG and TWIST that differ in protein structure, size, and individual functions. All of them are repressors of epithelial factors like E-cadherin and activate mesenchymal markers like Vimentin, Fibronectin or N-cadherin. Epithelial cells displaying apical–basal polarity are held together by tight junctions, adherens junctions, and desmosomes and are anchored to the underlying basement membrane by hemidesmosomes. They express three different polarity complexes that together with the junctional molecules maintain epithelial cell polarity. In the classical EMT, expression of EMT-TFs leads to inhibition of major components of these epithelial structures and concomitantly activates the expression of genes associated with the mesenchymal state. Cells gain front–rear polarity, display actin stress fibers, become motile and acquire invasive capacities. Notably, tumor cells very rarely switch to a completely mesenchymal phenotype, but fluently convert between various intermediate states displaying certain mesenchymal features but keeping partial sets of epithelial characteristics. Further, EMT is a reversible process. Mesenchymal cells can revert to the epithelial state undergoing MET. An important role in the execution of MET is played by microRNAs of the miR-200 and miR-34 families that are regulated in double-negative feedback loops with the EMT-TFs ZEB1/2 and SNAIL, respectively, that serve to reinforce either the epithelial or the mesenchymal state.

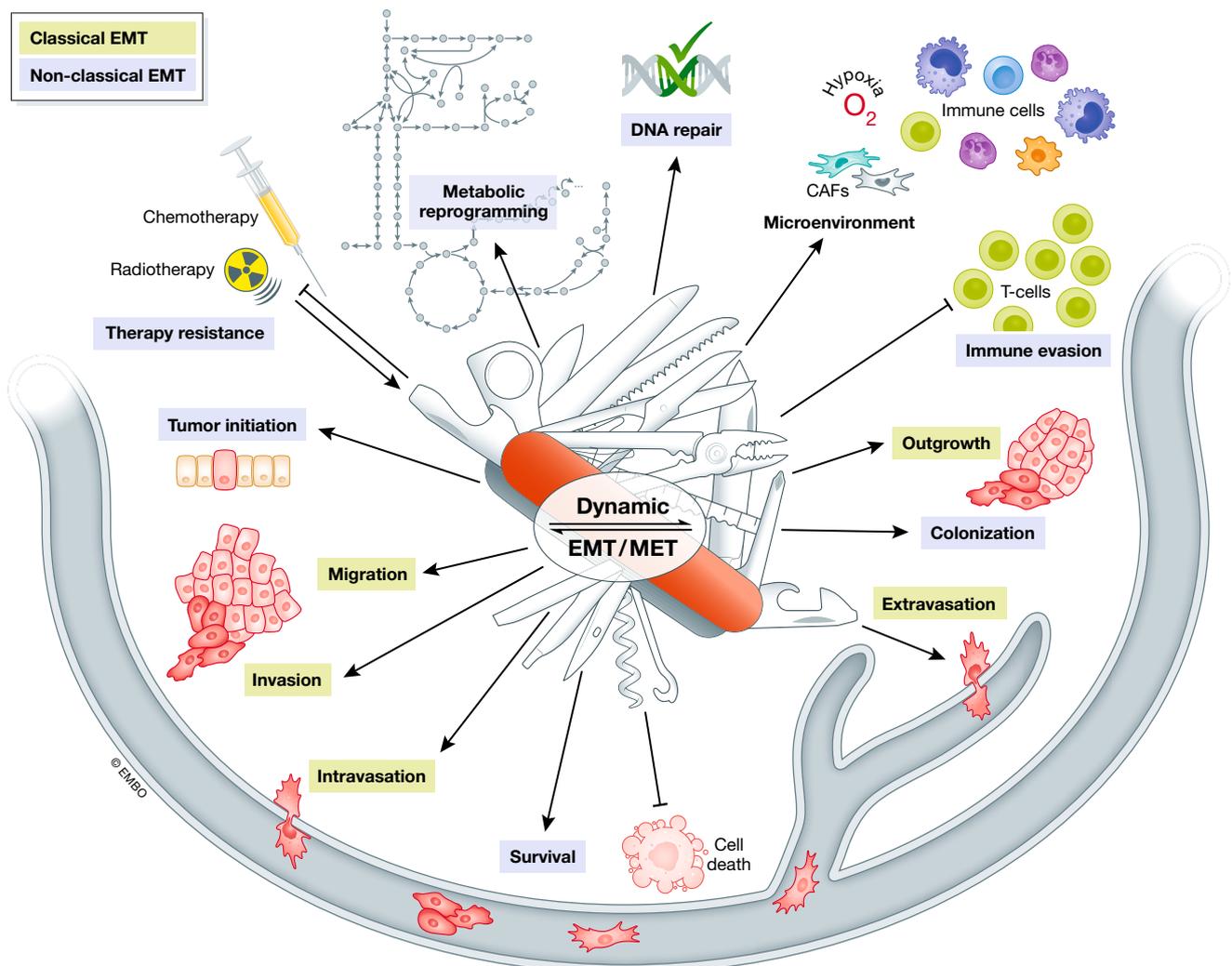


Figure 2. Dynamic EMT as a multi-tool for tumor progression.

Overview summarizing the multiple oncogenic EMT functions in the course of tumor progression. The classical EMT functions allow cancer cell to migrate, invade, intra- and extravasate blood and lymphatic vessels. At the distant sites, MET enables the outgrowth of macrometastases. The non-classical EMT traits support tumor initiation as well as metastatic colonization. Throughout the whole process of tumor progression, they help the cells to cope with changing conditions by metabolic reprogramming, enhanced survival via altered DNA repair and prevention of cell death, immune evasion and improved resistance to chemo- and radiotherapy. Importantly, EMT is not only supporting the cancer cells to handle changing environmental conditions, but is also induced by extracellular signals from, e.g., CAFs or immune cells in the microenvironment or by therapeutic approaches.

Classical/core EMT functions

Migration and invasion

In a normal epithelial tissue, the cells form continuous protective sheets that are crucial for their structural integrity. The connection between epithelial cells is mediated by different cell junction complexes including adherens junction, desmosomes, and tight junctions that seal the cells, are located apical to the adherens junctions to support epithelial polarity and constitute a barrier for solutes and water. This apical-basal polarity is fundamental for tissue function, and has been defined as “asymmetry” within cells and epithelial tissues. Polarity complexes, including the Par, the Crumbs, and the Scribble complexes, ensure the proper organization of apical versus basolateral domains in the epithelial cell (Huang *et al*, 2012). Of note, some of the genes that control epithelial cell polarity also regulate spindle orientation and division mode in stem cells (Martin-Belmonte & Perez-Moreno, 2011) (Fig 1B).

Elicited by the TME, the activation of EMT is fundamental for the transition toward malignancy, accompanied by substantial cellular changes on multiple levels. Cell–cell contacts become deconstructed through the repression of *CDH1*, encoding epithelial cadherin (E-cadherin), the main constituent of adherens junctions, and of genes coding for other epithelial junction molecules.

As a consequence of the disintegration of all epithelial junction complexes and of direct transcriptional repression of several members of the Crumbs and the Scribble complexes, apical–basal polarity is lost (Aigner *et al*, 2007; Moreno-Bueno *et al*, 2008; Spaderna *et al*, 2008; Lamouille *et al*, 2014) (Fig 1B). This coincides with profound cytoskeletal reorganization like apical constriction, the formation of actin stress fibers, and the conversion of cell morphology from cuboidal or columnar shapes to more spindle-like elongated forms with front–rear polarity, resulting in a gain of motility for the tumor cells (Moreno-Bueno *et al*, 2008). Newly formed actin-rich membrane protrusions like lamellipodia and filopodia support cell movement. To invade the surrounding tissues, e.g., TWIST or ZEB1 also induce the formation of invadopodia, specialized filopodia with proteolytic function (Yilmaz & Christofori, 2009; Eckert *et al*, 2011; Ridley, 2011; Sundararajan *et al*, 2015). This is supported by induction of matrix metalloproteases by EMT-TFs, essential for degradation of the basement membrane and the extracellular matrix of adjacent tissues (Miyoshi *et al*, 2004; Miyoshi *et al*, 2005; Huang *et al*, 2009) (Fig 1B). Additionally, EMT inducers prevent basement membrane synthesis by directly repressing the transcription of its components (Spaderna *et al*, 2006). All these classical EMT events cause the loss of epithelial integrity of the tissue, allow invasion and dissemination of cancer cells, and thus execute the first step of the metastatic cascade (Fig 1A).

MET: metastatic colonization and outgrowth

In 1990, Fearon and Vogelstein proposed a meanwhile classical genetic model for colorectal tumorigenesis. They described tumor development to be the continuous deterioration of initially normal epithelial cells toward greater malignancy driven by the stepwise accumulation of mutations and hypothesized that this process perpetuates during the last step of tumor progression from an established malignant carcinoma to distant metastases, implying that distant metastases are the most degenerated tissues of the

malignancy (Fearon & Vogelstein, 1990). However, all efforts to identify specific metastasis-associated mutations remained unsuccessful.

Rather, already twenty years ago, it was observed that metastases, compared with the de-differentiated nature of the invasion front of the primary tumor, exhibit a re-differentiated epithelial morphology, similar to the center of the primary tumor (Brabletz *et al*, 2001). These findings led to the hypothesis that de-differentiation and EMT during invasion is a transient condition and that an opposing process of epithelial re-differentiation or MET needs to be initiated and is advantageous for the formation of distant macrometastases (Figs 1A and 2). But why do metastases re-differentiate? Invasive, de-differentiated cancer cells were shown to be growth arrested, whereas proliferation was detected in re-differentiated metastasis, suggesting that EMT must be reversed in order to allow colonization and growth (Brabletz *et al*, 2001). This is supported by the fact that EMT-TFs can directly inhibit proliferation (Thiery *et al*, 2009) (Fig 3). There are also several publications confirming the relevance of MET for metastatic outgrowth (Chaffer *et al*, 2006; Korpál *et al*, 2011; Ocana *et al*, 2012; Tsai *et al*, 2012). Importantly, the occurrence of MET is in perfect accordance with the failure to identify EMT-causing mutations, but is attributed to the transcriptional and epigenetic regulation of the process.

Whereas EMT induction has been investigated in great detail (see below), less is known about the trigger of the reverse MET process. Is it just lack of EMT-inducing stimuli coupled to reduced expression of EMT-TFs? Several studies could show that knockdown of the EMT-TF *ZEB1* is sufficient to elicit MET *in vitro* in carcinoma cell lines of different entities (Eger *et al*, 2005; Spaderna *et al*, 2006; Spaderna *et al*, 2008) and that depletion of *Zeb1* in a genetic mouse model of pancreatic cancer fixes the tumor cells in an epithelial MET state (Krebs *et al*, 2017). Once expression of ZEB-family members (*ZEB1* and *ZEB2*) declines, their reduction is reinforced by a double-negative feedback loop with the miR-200 family of microRNAs. During EMT, ZEB factors transcriptionally repress the genes of all miR-200 family members. *Vice versa*, miR-200 family members inhibit expression of *ZEB1/2* at the post-transcriptional level (Fig 1B). Thus, the bidirectional transitions between EMT and MET states are potentiated by the ZEB/miR-200 circuit (Bracken *et al*, 2008; Burk *et al*, 2008; Wellner *et al*, 2009). This branch of MET was shown to be inducible via the tumor-suppressor p53, that directly activates miR-200s transcription (Kim *et al*, 2011). Upregulation of miR-200 family members has different consequences. It exerts both an invasion- and migration-inhibiting, thus tumor-suppressing function (Peter, 2009), and promotes metastatic colonization (Korpál *et al*, 2011). Importantly, these findings are not controversial, but highlight the dynamic nature of the EMT-MET process adapting the tumor cells to the respective conditions. Similar to the ZEB/miR-200 negative feedback loop, SNAIL and the miR-34 family constitute additional epithelial plasticity regulatory loops (Siemens *et al*, 2011; Diaz-Lopez *et al*, 2014) (Fig 1B). Of note, the described microRNAs target not only EMT-TFs themselves, but also genes like *BMI1*, *CD44*, *CD133*, *JAG1*, or *MYC* that are involved in tumor-relevant “non-classical” EMT functions like stemness and survival as discussed below (Brabletz & Brabletz, 2010; Brabletz *et al*, 2011).

Since the metastatic cascade is an extremely complex and slow multistep process that can take many years and requires very

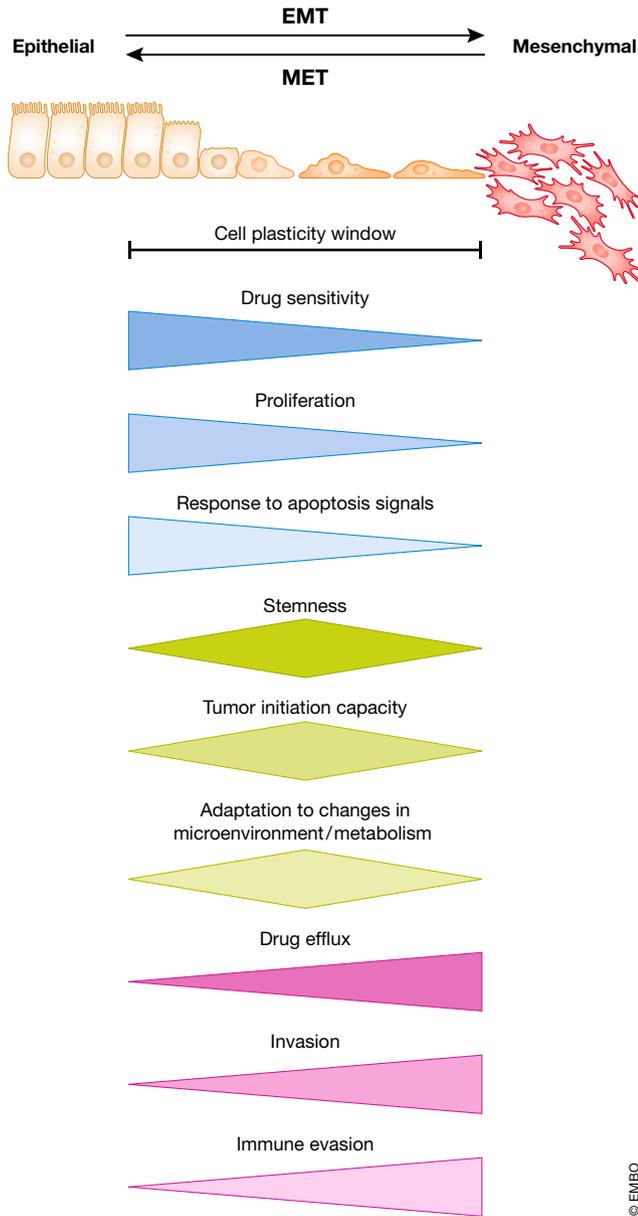


Figure 3. Cellular plasticity of tumor cells is governed by EMT and provided in a window of partial EMT.

Dynamic induction of EMT and MET changes cellular phenotypes of carcinoma cells. Drug sensitivity, proliferation, and response to apoptosis signals are highest in more epithelial states, whereas drug efflux, invasion, and immune evasion are highest in more mesenchymal states. A hybrid EMT state provides maximal stemness, tumor initiation capacity, and ability to adapt to environmental changes. Note that in extreme epithelial and mesenchymal states, features like stemness, tumor initiation, and colonization are lost.

different, sometimes apparently opposing traits from the tumor cells in a spatiotemporal manner, valid investigation remains challenging and must rely on mouse tumor models. Recently, several studies were focusing on the spatiotemporal regulation of EMT/MET using elegant mouse models. In 2012, Tsai et al (2012) demonstrated that EMT induction, in this case via TWIST, supports tumor cell

dissemination in a mouse model of skin cancer, but subsequent *Twist1* downregulation and MET was necessary for colonization and formation of macrometastases (Tsai et al, 2012) (Fig 3). Another study described the necessity of an EMT in the MMTV-PyMT mouse model of breast cancer to disseminate to the lung. Here, the EMT-state cancer cells at the metastatic niche activate local fibroblasts that in turn induce epithelial re-differentiation (MET) for outgrowth of macrometastases (del Pozo Martin et al, 2015). Further, Esposito and colleagues found that E-selectin mediated cell adhesion of bone vascular niche cells to cancer cells elicits MET and promotes bone metastasis (Esposito et al, 2019).

In summary, many reports support the occurrence and significance of MET in metastatic colonization and outgrowth (Fig 3). However, its regulation and detailed functions still need further investigation.

Partial EMT

EMT and MET have long been viewed as a binary switch between separate epithelial or mesenchymal cell populations. In the past, this narrow perspective challenged the concept of EMT as a major means in metastasis. One example for this is the analysis of Fischer et al using *Fsp1*-Cre driven lineage tracing in a genetic mouse model of breast cancer. Based on the finding that lung metastases mainly consist of tumor cells that had never switched to a full mesenchymal *Fsp1*+ phenotype, the authors concluded that EMT is not important for this process (Fischer et al, 2015).

However, nowadays it is accepted that, although reactivated in many cancer types, EMT is rarely fully executed in tumor cells, and end-stage markers such as Vimentin are often not expressed. The process is rather gradual and often remains incomplete, termed partial or hybrid EMT (Nieto et al, 2016; Pastushenko & Blanpain, 2019; Yang et al, 2020) (Fig 1B). Over the last years, more and more studies report *in vivo* detection of cancer cells carrying a combination of both epithelial and mesenchymal markers. Already in the 1990s, early analyses reported observations of cancer cells exhibiting only partial loss of E-cadherin during invasion (Mareel et al, 1992; Birchmeier & Behrens, 1994). Later, additional publications described circulating tumor cells (CTCs) with simultaneous expression of epithelial and mesenchymal markers (Yu et al, 2013). Similarly, partial EMT of cells within a tumor was identified by co-expression of epithelial (EpCAM+) and mesenchymal (Vim+) marker genes in an autochthonous murine prostate cancer model (Ruscetti et al, 2015). Partial EMT was also linked to metastasis via single-cell transcriptomics in head and neck cancer (Puram et al, 2017). Another group identified different tumor transition states occurring during EMT in cancer progression and introduced the term “hybrid” EMT (Pastushenko et al, 2018; Pastushenko & Blanpain, 2019; Pastushenko et al, 2021) (Fig 1B). Moreover, Bornes and colleagues demonstrated that *Fsp1*-Cre mediated lineage tracing as used by Fischer et al (2015) is incapable in detecting the majority of disseminating cells that are in a partial/hybrid EMT state (Fischer et al, 2015; Bornes et al, 2019). Importantly, this lack of full EMT does not mean that the process is not important. There is direct *in vivo* evidence that, e.g., the lack of *Zeb1* expression in a genetic mouse model of pancreatic cancer that traps the cancer cells in an epithelial

phenotype profoundly suppresses invasion and metastasis (Krebs *et al*, 2017). In mouse mammary carcinoma cells, SNAIL expression is crucial for *Zeb1* induction and metastatic dissemination (Ye *et al*, 2015). Similarly, in an oncogene-induced mammary tumor model, Xu *et al* (2017) found TWIST to be required for the expression of other EMT-TFs in a small subset of tumor cells to induce partial EMT and metastasis (Xu *et al*, 2017).

Interestingly, different modes of cancer cell invasion that involve EMT at various levels have been observed. Cancer cells can invade in groups forming rather epithelial clusters. Indeed, this type of “collective migration” might be even more common than single-cell dissemination, as supported by several studies using lineage tracing approaches and detection of clustered tumor cells in circulation (Friedl *et al*, 2012; Aceto *et al*, 2014; Cheung *et al*, 2016). Nevertheless, despite the epithelial appearance, either characteristic of a partial EMT is also detectable in these migrating cell clusters (Aiello *et al*, 2018), or the epithelial clusters follow mesenchymal “leader” cells that pave the way for their more epithelial “follower” cells (Matise *et al*, 2012; Chen *et al*, 2020) (Fig 1A).

Non-classical EMT features

Besides the classical features to drive changes in cellular phenotypes, EMT is also involved in regulating various additional aspects of tumorigenesis (Fig 2).

Regulation of stemness

Normal tissue homeostasis is dependent on the function of stem cells that serve as a source to replenish dying committed and terminally differentiated cells in a tissue. The observation that tumor heterogeneity is maintained after single tumor cell transplantations into mice, prompted to adapt normal stem cell biology also to tumors (Reya *et al*, 2001). Simplified, cancer cell stemness is measured by the capability of cell fractions to form tumors in mice. Strikingly, tumor initiation capacity is increasing with EMT activation by stem cell features provided by EMT-TFs within the cell plasticity window (Puisieux *et al*, 2014; Nieto *et al*, 2016; Shibue & Weinberg, 2017; Wilson *et al*, 2020) (Fig 3). Overexpression of *SNAIL*, *TWIST1*, and *ZEB1* in breast cancer is sufficient to increase the CD44⁺/CD24^{lo} stem cell pool, resulting in increased sphere forming capacity *in vitro*, as well as elevated tumorigenicity and metastasis *in vivo* (Mani *et al*, 2008; Morel *et al*, 2008). Moreover, SLUG determines stem cell states in the healthy mammary gland and converts luminal cells to stem cells in both healthy and tumor cell settings (Guo *et al*, 2012). Similar results were obtained for pancreatic cancer, where ZEB1 expression is a key determinant of cancer cell stemness and the reciprocal ZEB1/miR-200 feedback loop is controlling the expression of genes of stemness factors including *BMI1* and *SOX2* (Shimono *et al*, 2009; Wellner *et al*, 2009; Krebs *et al*, 2017). Similarly, in squamous cell carcinoma (SCC) TWIST is regulating *BMI1* to cooperatively repress *CDKN2A* (p16^{INK4A}) and promote tumor initiation capacities (Yang *et al*, 2010). Recently, the protocadherin *FAT1* was identified as one additional player in regulating EMT and stemness. *FAT1* is frequently inactivated resulting in increased tumor stemness and metastasis in a mouse model of SCC involving *CAMK2*, *CD44*, and *SRC* activities that induce nuclear

translocation of YAP1 and ZEB1 (Pastushenko *et al*, 2021). Interestingly, another important EMT promoting transcription factor, PRRX1, promotes EMT during development and in breast cancer cells, but its loss is required for cancer cell metastasis, thereby uncoupling EMT/migration from stemness features (Ocana *et al*, 2012). However, PRRX1 isoform switching is the driving force of invasion and metastasis in pancreatic cancer (Takano *et al*, 2016). Mechanistically, defined cellular states within the EMT cell plasticity realm correlate with gradually increasing efficiencies in metastatic seeding and invasion (Pastushenko *et al*, 2018), in line with the idea of partial/hybrid EMT states.

In addition to the regulation of tumor stemness and colonization (Shibue & Weinberg, 2009; Shibue *et al*, 2013; Nieto *et al*, 2016; Stemmler *et al*, 2019; Wilson *et al*, 2020), EMT is involved in driving tumor initiation and malignant transformation. *TWIST1* and *ZEB1* upregulation in RAS transformed mammary and bronchial epithelial cells is sufficient to unleash malignancy favoring formation of aggressive undifferentiated tumors (Morel *et al*, 2012; Liu *et al*, 2014b; Larsen *et al*, 2016). Moreover, EMT can induce reduction in KRAS dependency of tumor cells (KRAS addiction) and different ZEB1 thresholds drive KRAS-dependent tumor initiation and metastasis capacities (Singh *et al*, 2009; Liu *et al*, 2014b). The oncogenic effects of EMT-TF activities are even more evident by ectopic activation of *Zeb2* in the intestinal epithelium of transgenic mice. Elevation of ZEB2 generates invasive colorectal cancer in absence of cooperating genetic defects (Slowicka *et al*, 2020).

Therapy resistance

Loss of durable therapeutic efficacy and tumor relapse after initial successful treatment are major obstacles in the battle against cancer. Conventional therapy is favorably eliminating differentiated non-stem cell-like cells, but fails to deplete cancer cells with stem cell properties. EMT activation in these cells is controlling therapy resistance on multiple levels (Singh & Settleman, 2010; Shibue & Weinberg, 2017; Santamaria *et al*, 2019; Dudas *et al*, 2020). EMT gene signatures and acquisition of therapy resistance are strongly correlated, e.g., for standard and for targeted therapy with EGFR or PI3K inhibitors (Creighton *et al*, 2009; Farmer *et al*, 2009; Byers *et al*, 2013). For example, gemcitabine-resistant Panc1 pancreatic cancer cells exhibit EMT characteristics and become sensitized upon *ZEB1* knockdown (Wellner *et al*, 2009). Mechanistically, common routes toward drug resistance include increasing drug efflux or evading apoptosis and anoikis (Fig 3). The former is mediated by activating the expression of members of the ATP-binding cassette (ABC) transporter family, which are transcriptionally regulated by the EMT-TFs TWIST, SNAIL, and FOXC1 (Aller *et al*, 2009; Singh & Settleman, 2010; Saxena *et al*, 2011). SLUG and SNAIL contribute to evading therapy-induced apoptosis by interfering with p53 function, repression of the tumor-suppressor *PTEN*, or upregulation of the pro-survival protein BCL-XL (Vega *et al*, 2004; Escriva *et al*, 2008; Kurrey *et al*, 2009; Wu *et al*, 2015; Cao *et al*, 2016).

Experimentally transformed HMLER breast cancer cells with increased EMT features display about 10-fold increase in IC50 doses of many chemotherapeutics (Gupta *et al*, 2009; Singh *et al*, 2009; Singh & Settleman, 2010; Tulchinsky *et al*, 2019). In lineage tracing experiments, GFP-labeled mesenchymal tumor cells in PyMT tumors display high resistance to cyclophosphamide treatment (Fischer

et al, 2015). In non-small-cell lung cancer (NSCLC), activation of the AXL receptor tyrosine kinase in the course of EMT provides resistance to EGFR and PI3K inhibition, dependent on sustained KRAS activity (Singh et al, 2009; Sequist et al, 2011; Zhang et al, 2012; Byers et al, 2013; Tulchinsky et al, 2019). Furthermore, ZEB1 downregulation through HDAC class I inhibition or DNA demethylation resensitizes pancreatic cancer and osteosarcoma cells to chemotherapy (Meidhof et al, 2015; Ruh et al, 2021). Docetaxel-resistance of lung adenocarcinoma cells and paclitaxel-resistance of ovarian cancer cells can be reverted by blocking ZEB1 expression, providing increased survival of lung metastasis-bearing mice upon treatment (Ren et al, 2013; Sakata et al, 2017). In BRAF-mutant melanoma, ZEB1 is sufficient and only partly compensated by TWIST to promote an undifferentiated p75^{high} neural crest stem cell-like state that is characterized by resistance to MAPK and BRAF inhibitors (Richard et al, 2016; Shaffer et al, 2017; Rambow et al, 2018). In contrast, TWIST and SNAIL dampen the response to gemcitabine in an autochthonous mouse model of pancreatic cancer as *Snail* and *Twist1* knockout results in prolonged survival in gemcitabine-treated tumor mice (Zheng et al, 2015). Similarly, in NSCLC TWIST inhibition leads to sensitization to tyrosine kinase inhibitors of mutant EGFR (Yochum et al, 2019). In glioblastoma, the EMT-regulated shift toward a more stem-cell-like phenotype correlates with increased radioresistance. This is mediated by elevated STAT3 which is directly binding to the *SNAIL2* promoter for transcriptional activation (Lin et al, 2018). Collectively, EMT induction, increase in cancer stem cell features, and acquisition of therapy resistance are strongly connected and EMT-TFs are central players in orchestrating these processes.

EMT induced by therapy

As outlined before, one feature of EMT is to provide resistance to several treatments, including chemo-, radio-, and immunotherapy. Of note, also the therapy itself can induce an EMT phenotype with more aggressive features that involves activation of EMT promoting pathways via TGF β , NF- κ B, WNT, FGF, and EGF/HER2 (see below) (Terry et al, 2017; Redfern et al, 2018). Therapy-induced selection of cells with EMT characteristics, eventually displaying gain of ZEB1 and VIM, but repressed CDH1, MIR200C and MIR205 expression, was observed *in vitro* after long-term exposures to gemcitabine in BxPC3 pancreatic cancer cells and to Docetaxel in PC3 and DU-145 prostate cancer cells (Wellner et al, 2009; Puhr et al, 2012). Similarly, withdrawal of BRAF and MEK inhibition results in an enhanced motility and invasion through reactivation of ERK1/2 and AKT/mTOR *in vitro* (Norz et al, 2015). Treatment of mice suffering from CRC liver metastasis, with a vascular disruptive agent leads to a transient accumulation of nuclear β -catenin and upregulation of ZEB1, which reverts after treatment. This indicates that EMT induction might display adoption to cope with therapy-induced cellular stress (Fifis et al, 2013; Terry et al, 2017; Redfern et al, 2018). Supporting this hypothesis, inhibition of factors involved in therapy-induced DNA damage response (DDR), like CHK1 activation by ATR inhibitors, results in EMT and ZEB1 upregulation in CRC cells, reduced phosphorylation of CHK1 and decreased sensitivity to ATR inhibition (Song et al, 2018; Zhang et al, 2018). Observations in patients support a clinical relevance of EMT-associated pro-survival stress adaptation, exemplified by analysis of post-therapy specimens, e.g., in esophageal and

breast cancer after chemotherapy and in CRC after radiotherapy, showing decreased E-cadherin and elevated SNAIL and Vimentin levels (Creighton et al, 2009; Kawamoto et al, 2012; Hara et al, 2014).

Metabolic reprogramming

Metabolic rewiring is one important feature of cancer cells to cope with the excessive needs for energy and nutrients, enabling unlimited proliferation in conditions of rather restricted oxygen supply. In support of the metastasis cascade, reprogramming of glucose, lipid, and amino acid metabolism is tethered to activation of the EMT program and orchestrated by core EMT-TFs (Kang et al, 2019; Georgakopoulos-Soares et al, 2020; Sun & Yang, 2020; Bergers & Fendt, 2021). Cancer cell-mediated changes in metabolism are well described in glycolysis and triglyceride pathways, favoring the “Warburg effect”, i.e., energy-inefficient glycolysis even under normoxic conditions (Sun & Yang, 2020). This is another example how cancer cells adopt a developmental program of enhanced aerobic glycolysis that favors EMT and migration in the embryo (Youssef & Nieto, 2020). Upregulation of glucose transporters GLUT1 and GLUT3 is further promoting glycolysis. It was demonstrated in NSCLC that the increase in GLUT3 correlates with poor prognosis and EMT activation, mediated by activation of ZEB1 (Masin et al, 2014). On the contrary, metabolic abnormalities, e.g., in the tricarboxylic acid (TCA) cycle, can facilitate EMT. For example, loss of fumarate hydratase (FH) leads to aberrant methylation of a miR-200 family promoter region, resulting in enhanced expression of ZEB factors and EMT induction. Fumarate accumulation by repression of FH by the chromatin modifier LSH subsequently increases α -ketoglutarate (α -KG) levels. This affects IKK α -dependent EMT by promoting IKK α binding to and activation of EMT-associated gene promoters (He et al, 2016; Sciacovelli et al, 2016; Sciacovelli & Frezza, 2017; Georgakopoulos-Soares et al, 2020). Interestingly, ZEB1 is essential to allow metabolic plasticity. ZEB1 deficient pancreatic cancer cell lines are incapable of compensatory increasing glycolysis when oxidative phosphorylation is blocked, highlighting poor glycolytic reserve (Krebs et al, 2017). A systematic approach to categorize metabolic rewiring during EMT focused on gene expression alterations in high grade malignancies and identified a mesenchymal metabolic signature of 44 genes. Upregulation of this group of genes is correlated with a mesenchymal phenotype and is controlled by TWIST1 in mammary epithelial cells (Shaul et al, 2014).

In addition to carbohydrate pathways, EMT-associated changes in fatty acid, lipid and glycosphingolipid metabolisms are detected (Kang et al, 2019; Georgakopoulos-Soares et al, 2020; Hua et al, 2020; Bergers & Fendt, 2021). The morphological changes of cancer cells during EMT are accompanied by alterations in membrane fluidity and lipid composition. This requires the elevation of available fatty acids and is achieved for example by increased expression of the fatty acid translocase CD36. The presence of CD36 promotes EMT in HCC and is crucial for metastatic colonization of oral cancer (Nath et al, 2015; Pascual et al, 2017; Bergers & Fendt, 2021). Elevated free fatty acids (FFAs) lead to increased SRC and MMP9 activity that accelerates metastasis (Gubelmann et al, 2014; Jiang et al, 2020). In addition, the synthesis, storage and use of long-chain polyunsaturated fatty acids (PUFA) is elevated in the therapy-resistant EMT cell state. Consequently, the cells become dependent

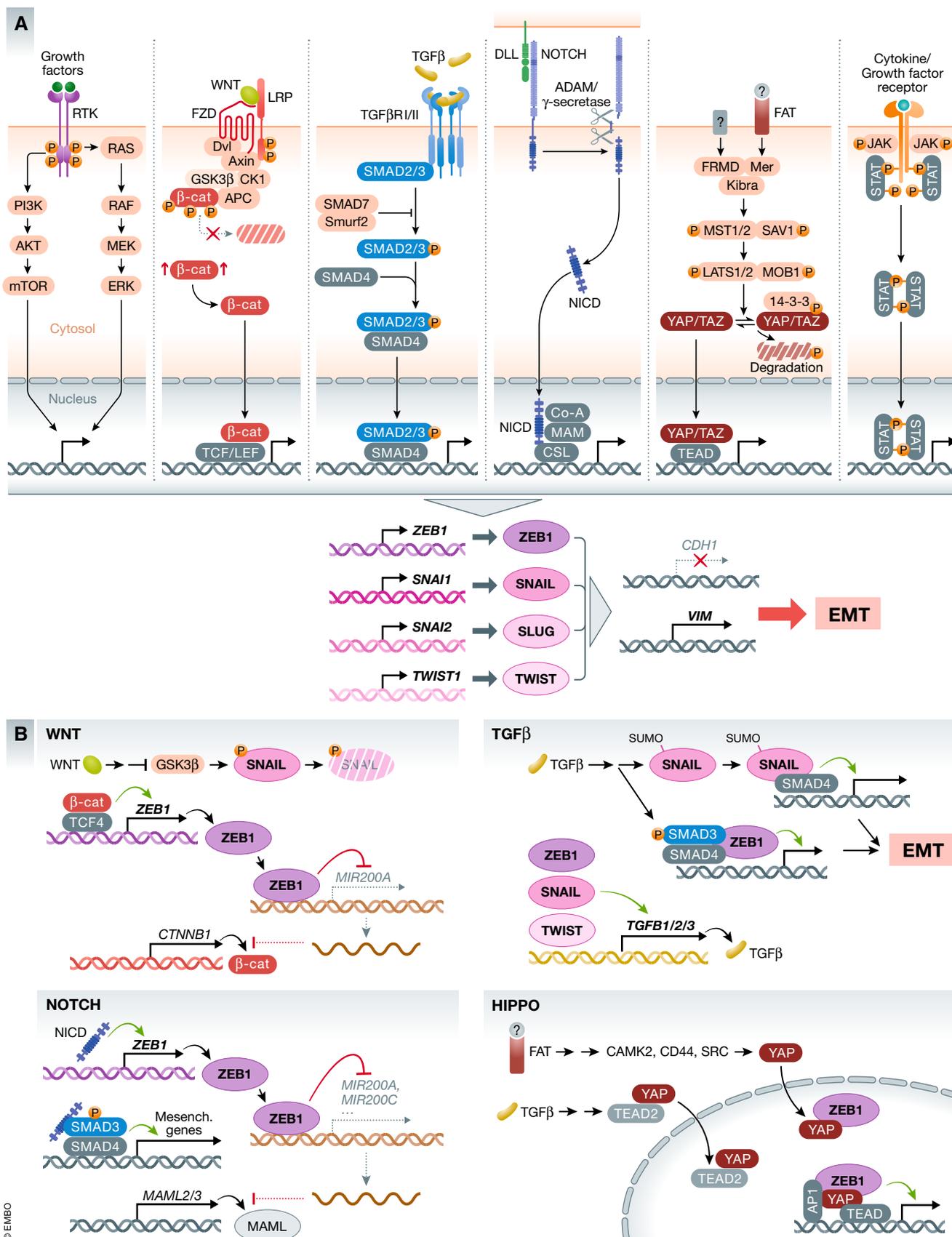


Figure 4.

Figure 4. Signaling pathways and their crosstalk during EMT induction.

(A) Canonical receptor tyrosine kinase pathways activated by EGF/EGFR, PDGF/PDGFR, HGF/c-Met and others, Wnt, TGF β , Notch, Hippo, and Cytokine signaling (e.g., IL6, TNF α) and their nuclear effectors are shown, which all promote expression of core EMT-TFs. Activation of EMT-TFs results in execution of the EMT program, exemplified by regulated *CDH1* and *VIM* expression. EMT is promoted by crosstalk of signaling pathways on multiple levels already in the cytoplasm (not shown). (B) Examples of EMT gene regulation of individual pathways and input from other signal transduction pathways. Effects on direct gene activation and repression are shown by green arrows and red block connections, respectively. For details, see text.

on elimination of resultant reactive lipid peroxides by Glutathione peroxidase 4 (GPX4) to evade the iron-dependent cell death pathway ferroptosis (Viswanathan *et al*, 2017). In summary, these analyses are a few examples demonstrating the reciprocal connection of EMT, cell plasticity, and metabolic reprogramming and how they synergize toward malignant progression (Fig 3).

EMT and immune evasion

During tumor progression, cancer cells develop a variety of strategies to withstand immunosurveillance and adaptive anti-tumor immunity that is induced by tumor neo-antigens. Most commonly pursued strategies include hiding the neo-antigen spectrum and formatting of an immunosuppressive environment, e.g., by hijacking negative immunological feedbacks referred to as “immune checkpoints” (Terry *et al*, 2017; Kudo-Saito *et al*, 2021). Interestingly, these unwanted processes often occur in strong correlation with EMT, and EMT-TFs have repeatedly been shown to co-regulate them directly or indirectly (Terry *et al*, 2017; Dongre & Weinberg, 2019; Jiang & Zhan, 2020; Kudo-Saito *et al*, 2021). For instance, defects in the immunoproteasome, that generates peptides for antigen presentation, were associated with a more mesenchymal phenotype in NSCLC (Tripathi *et al*, 2016). A study using the PyMT mouse model of breast cancer demonstrated a remarkable anti-correlation of mesenchymal expression profiles and MHC I expression (Dongre *et al*, 2017). Thus, human leukocyte antigen (HLA) class I complexes and major histocompatibility complex I (MHC I), required for antigen presentation, can apparently be deregulated by EMT-related mechanisms. Furthermore, utilizing breast cancer models *in vitro* and *in vivo* demonstrated that more mesenchymal tumor cells promote the generation and recruitment of regulatory T cells (T_{regs}) (Joffroy *et al*, 2010; Akalay *et al*, 2013; Dongre *et al*, 2017), which are an integral part of immune checkpoints able to inhibit cytotoxic T cells (CTL). In this regard, one of such intrinsic “brake” regulatory mechanisms of CTLs is activated by binding of their programmed cell death receptor (PD1) to its ligand PD-L1 provided by T_{regs} and myeloid antigen presenting cells. Tumor cells can hijack these immune checkpoints mainly by upregulation of *CD274* (PD-L1), which is strongly promoted by the EMT program (Zou *et al*, 2016; Terry *et al*, 2017; Jiang & Zhan, 2020; Kudo-Saito *et al*, 2021). In NSCLC, PD-L1 is activated by deregulation of the ZEB1/miR-200 axis in more mesenchymal cells (Chen *et al*, 2014; Jung *et al*, 2020). This regulation is likely independent of a general EMT program, since ZEB1 loss cannot be compensated by SNAIL, SLUG, or TWIST (Noman *et al*, 2017). Kudo-Saito showed that *SNAIL* expression in melanoma promotes induction of T_{regs} via Thrombospondin1 activation that results in resistance to immunotherapy (Kudo-Saito *et al*, 2009). Based on these findings, assessing the EMT signatures of, e.g., bladder cancer and melanoma may help to select patients for immune checkpoint blockers (Hugo *et al*, 2016; Kardos *et al*, 2016). In

summary, EMT cells evade T-cell anti-tumor immunity either via downregulation of antigen presentation or activation of immune checkpoints.

EMT and DNA integrity

Genomic instability is a common feature in cancer with evidence of contribution of EMT (Nieto *et al*, 2016; Yang *et al*, 2020) and has frequently been observed in pre-malignant mesenchymal cells *in vitro* as well as in tumors (Massague, 2012; Comaills *et al*, 2016; Caramel *et al*, 2018; Wang *et al*, 2019a). Although EMT stimuli are normally anti-proliferative, some cancer cells maintain cell doubling, perhaps at the expense of genome stability (Omabe *et al*, 2021). The involved mechanisms overriding the anti-proliferative signals and the generation of genomic aberrations are poorly understood. Usually, DNA repair, cell cycle checkpoints, senescence and cell death in response to endogenous (e.g., during DNA replication) and exogenous DNA damages (e.g., during chemotherapy) are orchestrated by the DDR, which is a conserved, multifactorial and context-dependent signaling network (Halazonetis *et al*, 2008; Omabe *et al*, 2021). Recent evidence suggests that EMT-TFs differentially affect the DDR. TWIST promotes chromosomal instability (CIN) by repressing mitotic checkpoint factors in colorectal cancer (CRC), whereas SLUG facilitates homologous recombination-directed DNA repair (HDR) during replication stress in mammary epithelial cells (Gross *et al*, 2019; Khot *et al*, 2020). Hence, regulation of the DDR by EMT-TFs may influence cancer treatment. In breast cancer cells, ZEB1 promotes radioprotection by driving the expression of the apical DDR kinase ATM via complex formation with p300 and PCAF at the *ATM* promoter as well as by stabilizing the mediator kinase CHK1 via sequestration of its deubiquitinase USP7 (Zhang *et al*, 2014; Zhang *et al*, 2018). In triple-negative breast cancer, ZEB1 was shown to directly repress *POLQ* (Pol θ) expression to prevent usage of the highly mutagenic alternative end joining (a-EJ or microhomology-mediated end joining) for the repair of endogenously occurring DSBs, thereby supporting the maintenance of genomic stability (Prodhomme *et al*, 2021). Intriguingly, during breast cancer development, the accumulation of CIN is apparently prevented, if oncogene activation had taken place in mammary stem cells, engaging a ZEB1-driven preemptive program with upregulation of the antioxidant MSR3. This mitigates oncogene-induced replication stress to overcome oncogene-induced senescence (OIS) in *TP53* wild-type cells (Halazonetis *et al*, 2008) (Morel *et al*, 2017). Along that line, SNAIL and TWIST regulate p53 and RB-dependent pathways to modulate OIS (Ansieau *et al*, 2008; Puisieux *et al*, 2014). In addition, SNAIL and SLUG alter the downstream DDR-driven cell death in MCF7 breast cancer cells by repressing pro-apoptotic effectors (Kajita *et al*, 2004). In summary, tumor cells utilize specific DDR-related functions of EMT-TFs to evade oncosuppressive mechanisms.

Box 1 Signaling pathways activating EMT in cancer

TGFβ

TGFβ is the major extrinsic signal of EMT in tumors, secreted by many cell types in the TME, but the role of TGFβ signaling in tumor initiation and progression is very complex as it shows a biphasic function with opposing effects. During tumor initiation, TGFβ promotes differentiation, cell cycle arrest, senescence and apoptosis in pre-malignant epithelial cells whereas during later stages TGFβ acts as inducer of EMT and promotes metastasis (Massague, 2012; Seoane & Gomis, 2017; Ramachandran *et al.*, 2018; Derynck *et al.*, 2021; Liu *et al.*, 2021) (Fig 4A). Moreover, TGFβ acts in a very context-dependent manner in crosstalk with other pathways, including Hippo, WNT, Notch, Hedgehog, ERK, p38 MAPK, PI3K/AKT and RHO-like GTPases to enhance their function (Bakin *et al.*, 2000; Zavadil & Bottinger, 2005; Hao *et al.*, 2019; Derynck *et al.*, 2021; Liu *et al.*, 2021) (Fig 4A). In transformed mammary epithelial cells, execution of TGFβ-mediated EMT depends on autocrine WNT activity and inhibition of these autocrine cues reduces tumorigenicity and metastasis *in vivo* (Scheel *et al.*, 2011). TGFβ induces mesenchymal genes like *VIM* and *FN1* as well as EMT-TFs *SNAI1*, *SNAI2*, *TWIST1* and *ZEB1* which form a feedback loop by activating TGFβ ligand expression to self-enforce and maintain TGFβ signaling (Dongre & Weinberg, 2019). Initial TGFβ receptor activation in breast or skin cancer results in transcriptional activation of *SNAI1*, combined with sumoylation of SNAIL at K234 fostering SMAD/SNAIL complex formation that promotes EMT (Hoot *et al.*, 2008; Vincent *et al.*, 2009; Gudey *et al.*, 2017) (Fig 4B). Similarly, ZEB proteins have SMAD-interacting domains and TGFβ, in crosstalk with MAPK signaling, utilizes SMAD3/SMAD4/ZEB1/2 complexes to regulate EMT-related gene expression (Postigo, 2003; Shirakihara *et al.*, 2007). Very strikingly, in pancreatic cancer, TGFβ-mediated EMT depends almost exclusively on ZEB1, since *Zeb1* knockout in KPC cells blocks more than 90% of TGFβ-induced alterations in gene expression, despite of *Snai1* upregulation during TGFβ treatment (Krebs *et al.*, 2017).

Notch

Like TGFβ, the Notch pathway is important for differentiation processes during development that in part involve EMT by *SNAI1* and *SNAI2* (Timmerman *et al.*, 2004; Niessen *et al.*, 2008). In cancer, a Notch-dependent EMT program is in part executed by cooperating with TGFβ signaling (Yuan *et al.*, 2014; Bray, 2016). Activation of the Notch pathway by Delta/Jagged ligands results in processing and release of the Notch ICD transducer that in turn interacts with SMADs, in addition to canonical RBP/J cofactor binding (Fig 4A and B). This crosstalk is essential for mesenchymal gene activation and regulates bone metastasis in NSCLC (Blokzijl *et al.*, 2003; Xie *et al.*, 2012; Liu *et al.*, 2014a; Yuan *et al.*, 2014). While in NSCLC Notch3 is crucial for invasion and EMT by directly regulating *ZEB1* transcription, in stratified squamous epithelia Notch3 promotes terminal differentiation. Oncogene-induced *NOTCH1* in SCC is activating *ZEB1* expression, which in turn downregulates *NOTCH3* to promote stem cell features and block differentiation (Liu *et al.*, 2014a; Natsuzaka *et al.*, 2017). Moreover, the *ZEB1*/miR-200 feedback loop further modulates Notch signaling activity as downstream Notch components including *MAML2/3* are miR-200 targets, resulting in enhanced Notch activation upon *ZEB1* induction (Brabletz *et al.*, 2011) (Fig 4B). Pancreatic cancer metastasis is promoted by a Notch-dependent inflammatory feedback loop that involves recruitment and activation of M2-like tumor-promoting macrophages and EMT via IL6/STAT3 signaling (Geng *et al.*, 2021).

Hippo/YAP/TAZ

Deregulation of the Hippo pathway was shown to promote tumorigenesis as well (Moroishi *et al.*, 2015; Ansari *et al.*, 2019) (Fig 4A). YAP/TAZ overexpression and activation is crucial to mediate EMT and cell plasticity in skin cancer and is sufficient to induce EMT in MCF10A non-cancerous mammary epithelial cells. YAP was shown to cooperate with KRAS for progression of pancreatic cancer and TAZ is a promoter of proliferation, EMT and stemness in breast and oral cancer (Zhang *et al.*, 2009; Shao *et al.*, 2014; Li *et al.*, 2015; Debaugnies *et al.*, 2018). The function of YAP/TAZ is dependent on the TEAD-binding domain and in breast cancer, TEAD2 is key to orchestrate EMT by regulating nuclear localization of YAP and TAZ (Lamar *et al.*, 2012; Diepenbruck *et al.*, 2014). In the very aggressive subgroup of Claudin-low breast cancer, YAP and ZEB1 interact to drive expression of a common target gene subset to promote EMT and metastasis (Lehmann *et al.*, 2016). Moreover, YAP forms a TEAD/YAP/AP1 ternary complex to regulate activity of distal enhancers, whereas ZEB1 and YAP are part of a multimeric complex with AP1 factors independent of direct ZEB1 DNA binding to drive gene expression on a genome-wide level (Zanconato *et al.*, 2015; Feldker *et al.*, 2020) (Fig 4B). Wang and colleagues demonstrated that in breast cancer, also TWIST is involved in YAP/TAZ-mediated EMT orchestrated by TWIST-dependent activation of the GPCR member *PAR1* (Wang *et al.*, 2020).

WNT

The role of WNT/β-catenin signaling in tumorigenesis and metastasis is highlighted by mutations of APC that preclude the control of the signaling competent cytoplasmic pool of β-catenin (Fig 4A). Moreover, the WNT target G-coupled receptor LGR5 labels stem cells in several organs as well as cancer stem cells in tumors (Clevers, 2006). Interestingly, LGR5 expression and WNT activity are crucial for disease progression, induction of EMT, stemness and metastasis, which is not provided by LGR5-negative tumor cells and depletion of *Lgr5⁺* cells in mice blocks metastasis (de Sousa e Melo *et al.*, 2017), though a *Lgr5⁻* state is detected in circulating CSCs (Fumagalli *et al.*, 2020). In breast cancer, reduction in WNT signaling by addition of secreted negative regulators of the pathways, like DKK1 or SFRP1 can promote reduction in migration and invasion, whereas inhibition of SFRP1, and thus elevated WNT signaling, can support an EMT phenotype and sensitize to TGFβ-induced EMT (Gauger *et al.*, 2011; Scheel *et al.*, 2011). Intracellularly, the WNT-induced block of GSK3β is not only promoting β-catenin cytoplasmic accumulation, but also stabilizes SNAIL (Zhou *et al.*, 2004). *ZEB1* is transcriptionally activated via β-catenin/TCF4 binding to the *ZEB1* promoter (Sanchez-Tillo *et al.*, 2011). Furthermore, increased ZEB1 in turn represses *MIR200A* expression which leads to de-repression of *CTNNB1* (β-catenin) thereby fueling this feedback loop (Su *et al.*, 2012; Liu *et al.*, 2013) (Fig 4B).

RTK signaling (induced by, e.g., EGF, FGF, IGF, HGF and PDGF)

Receptor tyrosine signaling has pleiotropic functions in proliferation and differentiation during development and homeostasis as well as in cancer initiation and progression. Growth factors like EGF, FGF, HGF and PDGF act via their cognate receptors and promote EMT either in support of TGFβ or alone via MAPK/ERK, PI3K/AKT and mTORC signaling (Lamouille & Derynck, 2007; Lamouille *et al.*, 2012; Dongre & Weinberg, 2019) (Fig 4A). The importance of RTK signaling is best described by the key function of RAS (*KRAS*, *HRAS*, *NRAS*) and *BRAF* oncogenes. One of them is mutated in most cancer types, leading to continuous pathway activation. *SNAI1* and *SNAI2* are direct targets of mutant *KRAS* and *BRAF* activities and *ZEB1/2* is regulated downstream by ERK2, but not ERK1 (Shin *et al.*, 2010; Makrodouli *et al.*, 2011). EGF induces EMT by upregulation of *SNAI1*, *TWIST1* and *ZEB1* in different cell lines through MAPK/ERK as well as JNK/STAT3 activation (Lu *et al.*, 2003). In particular, EGF is cooperating with other signaling pathways, e.g., IL6R/STAT3 and TGFβ, resulting in *CDH1* downregulation and enhanced cell motility and invasion (Lo *et al.*, 2007; Tian *et al.*, 2007; Uttamsingh *et al.*, 2008; Colomiere *et al.*, 2009). Phosphorylation of SMAD2/3 and nuclear co-localization with SNAIL was induced by activation of EGFR in MCF7 and MDA-MB-231 breast cancer

Box 1 (Continued)

cells (Kim *et al.*, 2016). EGF-induced EMT seems to require the function of the RNA-binding protein Musashi2 (MSI2) by interaction with ZEB1 to facilitate ZEB1-ERK/MAPK signaling in pancreatic cancer (Sheng *et al.*, 2020). Similar examples exist for activation of FGFR, IGFR, PDGFR and c-Met (HGF receptor) (Grotegut *et al.*, 2006; Yang *et al.*, 2006; Graham *et al.*, 2008; Devarajan *et al.*, 2012; Zhu *et al.*, 2016; Maehara *et al.*, 2017; Zheng *et al.*, 2019).

Cytokines and hypoxia

Several inflammatory cytokines, produced by cells in the TME, are contributing to cellular plasticity and an invasive phenotype (Fig 4A). Most prominently TNF α and IL6, but also IL1 β have critical functions and promote stemness, invasion and regulation of genes involved in EMT mediated by SNAIL, TWIST and ZEB1 as demonstrated in colon, breast and renal cell carcinoma (Bates & Mercurio, 2003; Sullivan *et al.*, 2009; Ho *et al.*, 2012; Li *et al.*, 2012; Miao *et al.*, 2014; Ieda *et al.*, 2019). IL6 signals through STAT3 that directly activates *SNAIL* and *ZEB1* expression, whereas TNF α facilitates TGF β and PI3K/AKT signaling that inhibits GSK3 β (Bates & Mercurio, 2003; Sullivan *et al.*, 2009; Ho *et al.*, 2012; Miao *et al.*, 2014) (Fig 4A). Interestingly, an autocrine feedback loop is generated in tumor cells to enhance an inflammatory phenotype, since SNAIL and ZEB1/2 are able to activate *IL6* expression in head and neck squamous carcinoma and breast cancer (Lyons *et al.*, 2008; Katsura *et al.*, 2017).

In addition to growth factors, chemokines and cytokines, hypoxia and HIF1 α are well known to induce EMT as well (Hapke & Haake, 2020). In this scenario, EMT is induced by inefficient neo-angiogenesis and deprivation of pericytes in the tumor (Hapke & Haake, 2020). Activated HIF1 α or HIF2 α directly or indirectly regulate *SNAIL*, *TWIST1* and *ZEB1* expression which fosters stemness and metastasis formation in bladder, ovarian, gastric and breast cancer (Yang *et al.*, 2008; Cooke *et al.*, 2012; Liu *et al.*, 2017; Hapke & Haake, 2020; Zhang *et al.*, 2020).

In summary, data that have been collected over decades provide a comprehensive picture in which EMT in tumorigenesis and metastasis is induced by integration of many pathways and growth factors. Moreover, synergism and the crosstalk of several signaling networks highlight that cellular plasticity in epithelial tumor cells renders the promotion of a cancer stem cell-like, immune evading, multi-resistant, invasive phenotype, able to adapt to changing conditions in the tumor and the metastatic niches. However, detailed mechanistic insight is still lacking to understand the complex crosstalks of signaling pathways that drive EMT and metastasis.

The tumor microenvironment: Stroma-tumor crosstalk to foster EMT

The TME is the source of many different signaling cues that have been implicated in EMT induction, including TGF β , WNT, RTK, Hippo, Notch and cytokine signaling (Box 1, Fig 4). The TME consists of extracellular matrix and non-transformed cells that are recruited to the tumor and adopt a variety of abnormal phenotypes instructed by tumor/stroma crosstalk. Cell types include fibroblasts, cells of the immune system, endothelial cells, adipocytes and neuronal cells (Fig 5). The complex crosstalk of the entity of these cell types and the ECM is providing pro- and anti-tumorigenic functions. Stroma composition has a strong impact on tumor aggressiveness and carries prognostic value (Hanahan & Weinberg, 2011; Jin & Jin, 2020). Moreover, individual stromal cell types are contributing to EMT and metastasis.

CAFs

Cancer-associated fibroblasts (CAFs) display a very heterogeneous population generated by reprogramming and expansion of either local tissue-resident or recruited fibroblasts from the periphery, including transdifferentiation of adipocytes, pericytes, monocytes, and mesenchymal stem cells (LeBleu & Kalluri, 2018; Sahai *et al.*, 2020). CAFs can be categorized into different subpopulations with specific functions and spatial organization (Lambrechts *et al.*, 2018; Elyada *et al.*, 2019; Sahai *et al.*, 2020). They serve as a primary source of EMT-inducing signaling molecules, like IL6, TNF α and TGF β , which promote a mesenchymal phenotype of tumor cells (Fig 5). Consequently, as an example they increase chemoresistance in NSCLC, regulate metastasis in ovarian cancer and promote oncogenic potential of adjacent epithelia in gastric squamous cell carcinoma (Bhowmick *et al.*, 2004; Yu *et al.*, 2014; Calon *et al.*, 2015; Shintani *et al.*, 2016; Zhao *et al.*, 2017; Goulet *et al.*, 2019). As demonstrated for

prostate cancer, the entry into TGF β -mediated EMT initiated by CAFs is dependent on changes in DNA methylation by DNMTA3A, silencing epithelial genes like *CDH1* and *GRHL2* (Pistore *et al.*, 2017). TGF β secretion by CAFs and EMT signatures in CRC are correlated with poor prognosis and promote metastasis (Calon *et al.*, 2015; Tauriello *et al.*, 2018). Interestingly, WNT signaling is involved in orchestrating CAF diversity and crosstalk to CRC tumor cells. In a subcutaneous organoid model, SFRP1 secretion induces a switch from contractile myCAFs to inflammatory iCAFs, accompanied by increasing EMT signatures in tumor cells (Mosa *et al.*, 2020). Moreover, CAFs can impinge on anti-tumor immunity and on success of anti-CSFR1 immunotherapy (Kumar *et al.*, 2017; Tauriello *et al.*, 2018), supported by the finding that collagen 1 (COL1) deposition by myCAFs is crucial to augment an immunosuppressive TME (Chen *et al.*, 2021).

TAMs

Macrophages in the TME are contributing to EMT on multiple levels. In a simplified model, macrophage-tumor crosstalk generates a variety of tumor-associated macrophage (TAM) phenotypes, with an M1-like state promoting elimination of immunogenic tumor cells and a contrasting pro-tumorigenic M2-like state (Gonzalez *et al.*, 2018). In crosstalk with other cell types and in the presence of TGF β , metastasis is induced by CD4⁺ T-cell derived IL4 and macrophage-derived WNT7B/EGF as well as CSF1 from tumor cells. These ligands induce co-migration of tumor cells and macrophages toward blood vessels. TAMs near blood vessels create “the tumor microenvironment of metastasis” enabling tumor cell intravasation (Joyce & Pollard, 2009; Noy & Pollard, 2014; Gonzalez *et al.*, 2018) (Fig 5). TAMs can also directly express and secrete TGF β and cytokines to induce EMT. In F9 teratocarcinoma, NMuMG cells and hepatocellular carcinoma (HCC), intratumoral or cocultured macrophages are secreting TGF β to promote EMT (Bonde *et al.*, 2012; Fan *et al.*, 2014). In CRC, EMT is further accelerated by TNF α -secreting

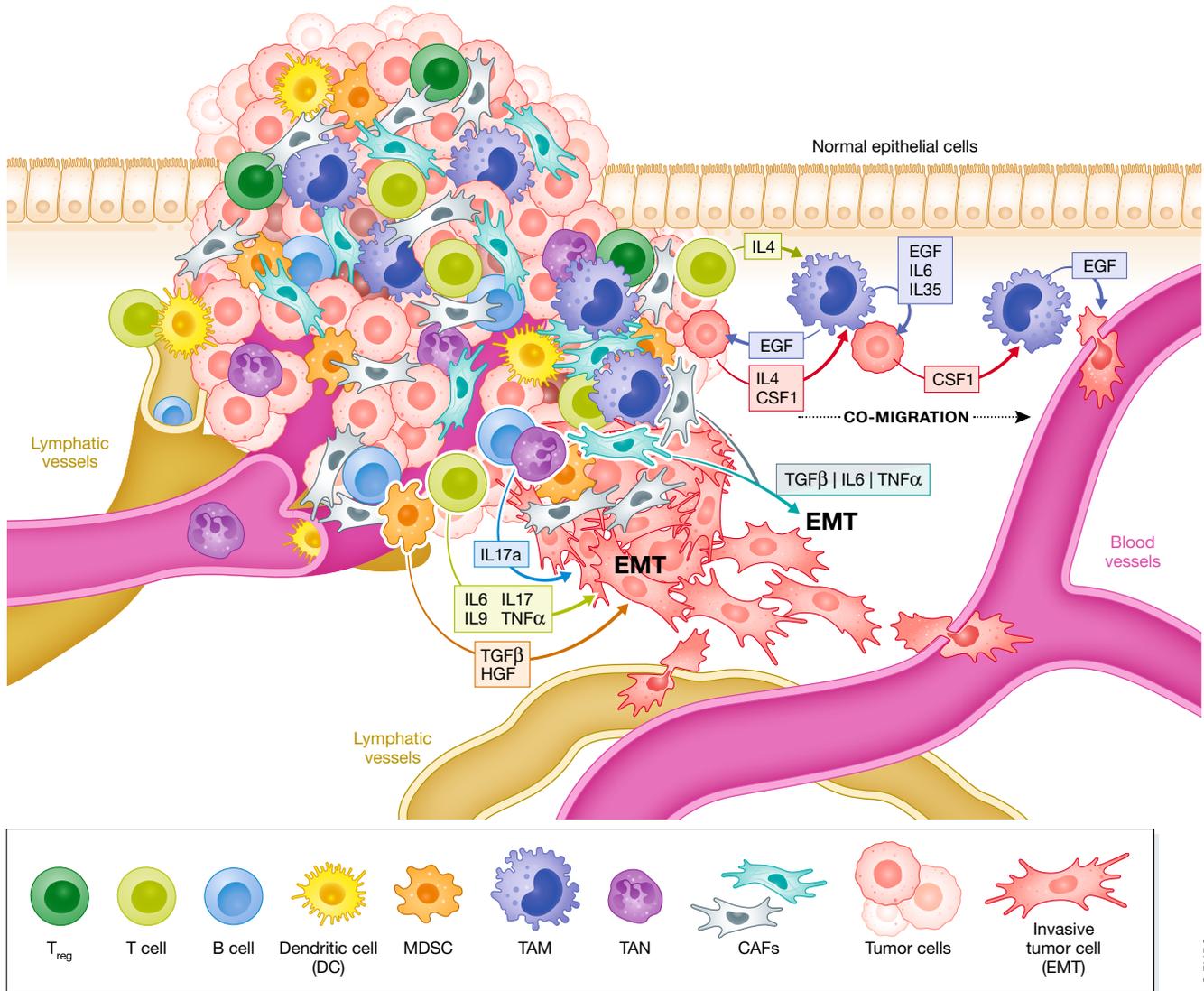


Figure 5. Cell types in the tumor microenvironment and contribution to EMT.

Cells of the hematopoietic lineage, fibroblasts and others contribute to the TME. Exemplified modes of action and cytokines secreted from individual cell types are depicted which promote EMT, migration, invasion, and intravasation of tumor cells into blood vessels. EMT induction and co-migration can be induced by TAMs and specific lymphocyte/tumor cell crosstalk.

macrophages, inducing p38 MAPK signaling in tumor organoids (Bates & Mercurio, 2003). Moreover, in renal clear cell carcinoma, NSCLC, colon and breast cancer IL6 and IL35 are produced by macrophages and promote EMT by prostaglandin/ β -catenin and JAK2-STAT6-GATA3 signaling, respectively (Zhang *et al*, 2019b; Sun *et al*, 2020) (Che *et al*, 2017; Lee *et al*, 2018).

MDSCs

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous cell population originating from neutrophils and monocytes. They are expanding in cancer patients and are involved in immune cell suppression, specifically of T cells. Accordingly, they promote tumor cell immune escape and block cytotoxic T-cell activity

(Gabrilovich & Nagaraj, 2009; Gabrilovich, 2017). Moreover, they directly induce EMT via release of different growth factors and cytokines, including TGF β and HGF, as well as the high mobility group protein B1 (HMGB1) which acts through toll-like receptors (TLRs) and leads to induction of *Snail1*, *MMP7* and *NFKB1* (NF- κ B) (Trovato *et al*, 2020) (Fig 5). At the primary tumor, specific monocytic CXCR2-expressing MDSCs (mMDSCs) promote EMT and they are recruited by breast cancer cells via secretion of CXCL1/2, whereas MET at pulmonary metastatic sites is induced by granulocytic MDSCs (gMDSCs) (Ouzounova *et al*, 2017; Zhu *et al*, 2017; Taki *et al*, 2018; Trovato *et al*, 2020). Of note, MDSCs become activated and expand upon operational stress during primary breast cancer resection and start to secrete TGF β , VEGF and IL10 to

promote lung metastasis in a 4T1 orthotopic breast cancer model (Ma *et al*, 2019).

TANs

Neutrophils are promoting metastasis at the pre-metastatic niche in lungs and liver by suppressing T cells (Gonzalez *et al*, 2018). In addition, they are involved in promoting EMT at the primary site. Akin to TAMs, tumor-associated neutrophils (TANs) can polarize between extreme states “N1” TANs with anti-tumor and pro-immune functions, induced by IFN- β , and “N2” TANs with pro-tumorigenic and pro-metastatic functions. “N2” TANs are induced by TGF β (Gonzalez *et al*, 2018; Masucci *et al*, 2019; Wu & Zhang, 2020). Neutrophils in the stroma of gastric cancer (GC) produce IL17a and CXCL5 both promoting EMT, indicated by upregulation of *VIM* and *ZEB1*, whereas *CDH1* is repressed (Fig 5). Anti-IL17a treatment *in vitro* blocks EMT in neutrophil-GC co-cultures (Li *et al*, 2019; Mao *et al*, 2020; Wu & Zhang, 2020). In MCF7 breast cancer cells, EMT is induced by TIMP-1 expression secreted by neutrophils. TAN-MCF7 interaction creates an enforcing feedback-loop as MCF7 cells with a more mesenchymal phenotype activate *TIMP-1* expression in neutrophils by CD90 during direct cell contact. Blocking CD90 reduces the number of metastasis in a 4T1 orthotopic injection model in mice (Wang *et al*, 2019b; Wu & Zhang, 2020).

T cells

CD4⁺ and CD8⁺ T cells are the most prominent effectors of anti-tumor immune response. High levels of T-cell infiltration usually correlate with better prognosis; however, their activity is dependent on immune regulatory and suppressive mechanisms including T_{reg} activities (Joyce & Pollard, 2009; Gonzalez *et al*, 2018). Moreover, some examples demonstrate direct contribution of T cells to EMT. In the presence of isolated effector CD4⁺ T cells, pancreatic cancer cells show upregulation of *VIM*, *L1CAM* and *ZEB1*, combined with a more migratory phenotype, which was reverted by blocking IL6 and TNF α (Goebel *et al*, 2015). In breast cancer, also CD8⁺ cytotoxic T cells induce EMT, specifically during relapse and loss of a HER2/neu antigen-mediated response, resulting in a more stem cell-like phenotype with increased therapy resistance (Kmieciak *et al*, 2007; Santisteban *et al*, 2009). In lung cancer, specific T-lymphocyte subsets induce EMT in co-culture experiments via IL9 and IL17 secretion and block of IL9/IL17 reduced EMT and metastasis (Salazar *et al*, 2020) (Fig 5).

Therapeutic options to target EMT

As outlined in the previous chapters, various aspects of EMT are contributing to poor outcome, including resistance to treatment. Since therapeutic intervention with standard care, targeted therapy, irradiation, and surgery is often promoting EMT, major benefit is expected from therapeutic approaches that prevent or reverse the fatal effects of EMT. Standard treatment for targeting differentiated highly proliferative cancer cells combined with specific therapy to either block/revert an EMT/stemness/therapy-resistant state or to target specific, yet unidentified, vulnerabilities of such exquisitely plastic cells seems advantageous. Several molecules to target EMT have been or are currently in clinical trials whereas others are already in use. These include blocking of upstream pathways which

promote tumorigenesis also beyond EMT by ligand-neutralizing antibodies, decoy receptors or inhibitors to block TGF β , NF- κ B, EGFR, c-MET, WNT and Notch signaling (comprehensively reviewed in (Redfern *et al*, 2018; Dudas *et al*, 2020; Jonckheere *et al*, 2021).

Due to difficulties in targeting transcription factors, i.e., EMT-TFs, directly, another promising approach is to apply modified synthetic miRNAs that interfere with EMT-TFs on a post-transcriptional level. Preclinical data demonstrate that miR-34 and miR-200s administration will attenuate SNAIL and ZEB1 protein levels, respectively, and inhibit stemness and metastasis (Pecot *et al*, 2013; Cortez *et al*, 2014). Liposomal miR-34 (MRX34) is already used in clinical trials with effects on tumor growth and metastasis (Zhang *et al*, 2019a).

Another interesting approach to block cell plasticity and to resensitize tumor cells to standard therapy is to induce re- or transdifferentiation. Interfering with chromatin remodeling by using HDAC inhibition or DNA-demethylating agents has been proven valid methods to induce differentiation. In pancreatic cancer, the application of the HDAC class I inhibitor mocetinostat results in de-repression of ZEB1-inhibiting miRNAs, ZEB1 downmodulation, MET, and resensitization to gemcitabine in mice (Meidhof *et al*, 2015). Reactivating expression of silenced genes has a similar effect in osteosarcoma. Demethylation of the imprinted *DLK-DIO3* locus by 5-Azacytidine reactivates expression of ZEB1-silencing miRNAs, induces osteogenic and adipogenic differentiation, and sensitizes to doxorubicin treatment (Ruh *et al*, 2021). The idea of transdifferentiation was nicely demonstrated by Ishay-Ronen *et al*, showing that EMT-derived breast cancer cells can be differentiated into post-mitotic functional adipocytes by rosiglitazone *in vivo*, resulting in reduced metastasis (Ishay-Ronen *et al*, 2019). Another group of molecules that induce re-differentiation and drug sensitivity includes compounds generated for different purposes, like metformin, used for type2 diabetes, or herbal components, like curcumin (Kothari *et al*, 2014; Dudas *et al*, 2020; Jonckheere *et al*, 2021). Metformin leads to reduction in SNAIL, TWIST, and ZEB1 levels, and curcumin inhibits EMT by downregulation of NF- κ B/SNAIL in breast cancer and reactivation of epithelial miRNAs (Kothari *et al*, 2014; Dudas *et al*, 2020).

With all these promising approaches to block and reverse EMT, it is important to consider potential adverse effects. Although EMT is harmful during tumorigenesis and EMT-TFs contribute to poor prognosis, they play very important physiological roles. EMT is involved in wound closure and repair after injury and EMT-TFs have important functions in stem cell homeostasis and differentiation, e.g., in melanocytes, muscle, bone and the nervous system, as well as in innate and adaptive immune responses. Accordingly, global interference with the context-dependent favorable actions of EMT-TF by special therapies may well affect normal tissue homeostasis. Likewise, miRNA-based applications will simultaneously regulate other targets with unpredicted outcome. On the contrary, therapeutic intervention that favors MET, might promote colonization and metastasis of circulating tumor cells, leading to opposing effects. Hence, a very careful evaluation and examination of adverse effects is needed to promote such approaches in the clinic. Moreover, we are only beginning to understand the complex nature of the multi-tool EMT in cancer and integration of aspects of multiple pathways and of TME diversity are the main challenges toward a more comprehensive understanding of metastasis for identifying novel treatment approaches.

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Conflict of interest

The authors declare that they have no conflict of interest.

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